AD_____

Award Number: W81XWH-09-1-0439

TITLE: Tumorigenic Potential of Transit-Amplifying Prostate Cells

PRINCIPAL INVESTIGATOR: Sarki Abba Abdulkadir

CONTRACTING ORGANIZATION: The Vanderbilt University
Nashville, TN 37203

REPORT DATE: June 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-Jun-2010	Annual	1 Jun 2009 – 31 May 2010

4. TITLE AND SUBTITLE

Tumorigenic Potential of Transit Amplifying Prostate Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0439

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Sarki Abdulkadir

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Vanderbilt University

Nashville, TN 37203-6876

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
And Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT An understanding of t he identit y of th e target cell for prostate cancer develo pment is essential to the development of accurate diagnostic, prognostic and therapeutic approaches. We hy pothesized that that loss of the prostate tumor suppressor Nkx3.1 sensitizes cells to prostate tumorigenesis by arresting cells in a precursor transit amplifying state that is more susceptible to tum origenesis by oncogenes such as c- MYC that can im part self-renewal p otential. Further we hypothesize that these alterations in the ce llular differentiation state are controlled by global alter ations in chro matin whic h influence gene expressio n. Our specific goals were to test the susceptibility of Nkx3.1-null transit am plifying cells to transformation and tu morigenicity in vi tro and in vivo in res ponse to MY C and to exa mine the basi s of the dysregulation of histone deacety lase enz ymes and their functional consequences in Nkx3.1-null cells. To date, we have established a tissue recombination sy stem for prostate regene ration using prim ary m ouse prostate cells . Using this sy stem, we have obtained evidence that loss of Nkx3.1 cooperates with MYC expression in prom oting prostate tumorigenesis in vivo. These results indicate that Nkx3.1-deficient cells are sensitive to MYC-induced tumorigenicity.

15. SUBJECT TERMS Prostate cancer, stem cell, Nkx3.1 tumor suppressor, MYC oncogene

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	8	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

Introduction…….. 3

BODY…….. 3-4

Key Research Accomplishments…….. 4

Reportable Outcomes…….. 4

Conclusion…….. 4

References…….. 4

Appendices…….. 5-7

INTRODUCTION

Recent advances in our understanding of the parameters associated with tumor aggressiveness, response to therapy, resistance and relapse point to a need for a better delineation of the cellular origin of human cancers. In the prostate, cells are organized in a hierarchical manner, with rare populations of stem cells at the top of the hierarchy. These give rise to more differentiated progenitors with high proliferation potential but limited self-renewal termed "transit amplifying cells", which in turn are believed to give rise to the terminally differentiated cells whose primary function is secretory (Matusik et al., 2008; Xin et al., 2007). Knowing the target cell type for prostate tumorigenesis is crucial for developing efficient diagnostic, prognostic and therapeutic approaches that can lead to the elimination of the tumor (Dirks, 2008; Lawson and Witte, 2007). NKX3.1 encodes a homeodomain transcription factor whose expression is androgen-dependent and largely restricted to the luminal epithelial cells of the prostate (Abate-Shen and Shen, 2000; Abdulkadir, 2005). Functional studies indicate that the primary defect in Nkx3.1 null mice is a failure of transit amplifying cells to undergo terminal differentiation and exit the cell cycle in a timely manner (Abdulkadir et al., 2002; Magee et al., 2003). An oncogene frequently overexpressed in human prostate cancer, MYC, has been implicated in stem cell function. MYC is sufficient to activate a transcriptional program important for cancer stem cell function in multiple epithelial cancers suggesting that it can promote self-renewal (Wong et al., 2008). Thus, our main goal is to test the hypothesis that progenitor transit amplifying cells that accumulate in Nkx3.1-null mice are sensitive to tumorigenic induction by MYC. We will also test the role of the histone deacetylase HDAC1 in regulating chromatin modification, cell cycle exit and tumor susceptibility of Nkx3.1-deficient cells.

BODY

We will report on studies outlined in Aim 1 of the proposal which cover Months 1-12.

Aim 1: To test the susceptibility of Nkx3.1-null transit amplifying cells to transformation and tumorigenicity by MYC in vitro and in vivo using primary and established cell lines. The *in vivo* studies will use a facile tissue recombination system.

Task 1: Isolate prostate epithelial cells from Nkx3.1+/+ and Nkx3.1-/- mice, infect with MYC-expressing or GFP control lentivirus. Generate and analyze numbers and sizes of prostaspheres formed by growth in matrigel. Serially passage prostaspheres by trypsin digestion; assess rate of formation of secondary and tertiary spheres in matrigel as an indication of self-renewal potential. 20 mice. Months 1-3. ***Task 2:*** Molecular characterization of prostaspheres using RT-PCR and immunohistochemistry for markers of differentiation. In vitro assays of transformation on lentivirus-infected mouse primary prostate epithelial cells: focus formation and soft agar assays. 20 mice. Months 1-3.

Task 1&2 Results: We have successfully established conditions for generating prostaspheres in matrigel from primary prostate cells isolated from wild type and Nkx3.1 knockout mice (Fig. 1). The sphere cells express the prostate epithelial marker keratin 8, K8. Quantitation of primary sphere number and size did not show a significant difference between wild type and knockout (Fig. 2). However, secondary spheres generated by serial passaging show a trend for reduced number of spheres from the Nkx3.1 knockout mice (Fig. 3). Additional experiments are underway to determine if this difference is statistically significant. Additional support for a change in self renewal due to lack of Nkx3.1 is provided by gene expression analysis using quantitative RT-PCR (fig. 4). The stem cell related genes Lgr5 and Bmi1 (Wong et al., 2008) in particular showed reduced expression in KO cells.

Task 3: In vivo tissue recombination experiments. Primary mouse prostate epithelial cells from Nkx3.1+/+ and Nkx3.1-/- mice isolated and infected with MYC and GFP-control lentiviruses. Cells are recombined with fetal rat urogenital mesenchyme and implanted in severe combined immunodeficient mice. 86 mice, 15 rats (Months 1-12).

Task 3 Results: We isolated adult prostate epithelial cells from adult Nkx3.1+/+ and Nkx3.1-/- mice and infected them with lentivirus co-expressing MYC and YFP (yellow fluorescent protein) or YFP alone. The

prostate epithelial cells were then recombined with fetal rat urogenital mesenchyme (UGM) in collagen and implanted under the kidney capsules of immunodeficient mice to regenerate prostates (Fig. 5A,B). Six weeks later, the grafts (n=3 each) were harvested and characterized by histology. We found that Nkx3.1+/+;YFP grafts formed normal looking prostate glands, while Nkx3.1-/-;YFP grafts showed prostatic epithelial hyperplasia consistent with the phenotype of Nkx3.1-/- mice (not shown). Nkx3.1+/+;MYC grafts on the other hand consisted of glands containing high-grade PIN (HGPIN) lesions while Nkx3.1-/-;MYC grafts showed HGPIN with microinvasive carcinoma as confirmed by focal loss of smooth muscle actin as well as p63 basal cell staining (Fig. 5C). The Nkx3.1-/-;MYC grafts also showed higher proliferation without a concomitant increase in apoptosis (Fig. 5D). The results of this pilot study with a single time point indicate cooperativity between Nkx3.1 loss and Myc overexpression in prostate cancer. Additional tissue recombination experiments are ongoing to generate more grafts.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of prostate sphere assay
- Demonstration that loss of Nkx3.1 does not affect primary prostate sphere number or size
- Demonstration of alterations in stem cell genes (Lgr5 and Bmi1) in Nkx3.1 KO prostate cells
- Demonstration that loss of Nkx3.1 cooperates with MYC expression in prostate tumorigenesis in vivo

REPORTABLE OUTCOMES:

None

CONCLUSION:

We have found that Nkx3.1 loss may affect self-renewal of prostate epithelial cells in in vitro prostate sphere assays. Furthermore, loss of Nkx3.1 affects expression of some stem cell genes. These interesting findings may indicate a defect in Nkx3.1 deficient cells that has to be overcome for tumorigenesis to proceed, hence the need for cooperation with other mutations such a Myc expression. This is supported by out in vivo tissue recombination data with Nkx3,1 knockout cells. These findings need to be confirmed. We have found that secondary sphere assays with Nkx3,1 knockout cells is difficult due to loss of viability of the cells, necessitating the use of more mice than anticipated. We have also had some failed grafts in in vivo studies due to loss of cell viability. These technical difficulties have been overcome. Going forward, we are generating more grafts to confirm our findings.

REFERENCES

Abate-Shen, C., and Shen, M. M. (2000). Molecular genetics of prostate cancer. Genes Dev *14*, 2410-2434.

Abdulkadir, S. A. (2005). Mechanisms of prostate tumorigenesis: roles for transcription factors Nkx3.1 and Egr1. Ann N Y Acad Sci *1059*, 33-40.

Abdulkadir, S. A., Magee, J. A., Peters, T. J., Kaleem, Z., Naughton, C. K., Humphrey, P. A., and Milbrandt, J. (2002). Conditional loss of nkx3.1 in adult mice induces prostatic intraepithelial neoplasia. Mol Cell Biol *22*, 1495-1503.

Dirks, P. B. (2008). Cancer's source in the peripheral nervous system. Nat Med *14*, 373-375.

Lawson, D. A., and Witte, O. N. (2007). Stem cells in prostate cancer initiation and progression. J Clin Invest *117*, 2044-2050.

Magee, J. A., Abdulkadir, S. A., and Milbrandt, J. (2003). Haploinsufficiency at the Nkx3.1 locus. A paradigm for stochastic, dosage-sensitive gene regulation during tumor initiation. Cancer Cell *3*, 273-283.

Matusik, R. J., Jin, R. J., Sun, Q., Wang, Y., Yu, X., Gupta, A., Nandana, S., Case, T. C., Paul, M., Mirosevich, J., *et al.* (2008). Prostate epithelial cell fate. Differentiation.

Wong, D. J., Liu, H., Ridky, T. W., Cassarino, D., Segal, E., and Chang, H. Y. (2008). Module map of stem cell genes guides creation of epithelial cancer stem cells. Cell Stem Cell *2*, 333-344.

Xin, L., Lukacs, R. U., Lawson, D. A., Cheng, D., and Witte, O. N. (2007). Self-renewal and multilineage differentiation in vitro from murine prostate stem cells. Stem Cells *25*, 2760-2769.

APPENDICES

None

SUPPORTING DATA

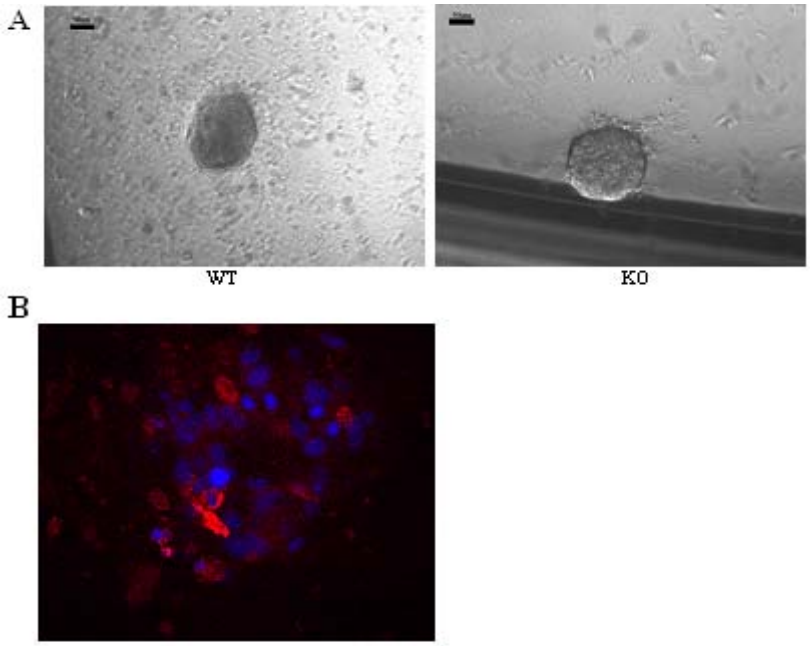
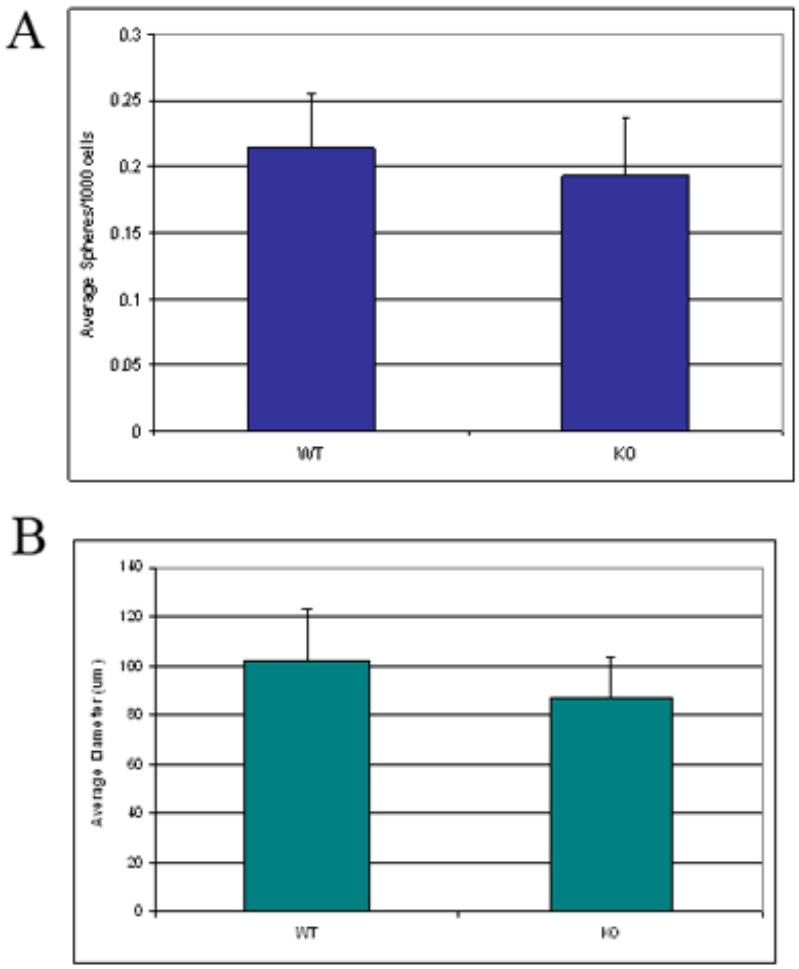


Fig. 1: Prostaspheres from Nkx3.1 wild type (WT) and knockout mice (KO). A) Primary prostate epithelial cells were isolated from adult (8week) old mice of the indicated genotypes. Cells are grown in matrigel and images captured after 10 days. B) Fluorescence confocal microscopy demonstrates that these spheres express luminal epithelial marker K8. (Nucleus -blue, K8 - red)

Fig. 2: Loss of Nkx3.1 does not affect primary sphere formation. A) Formation of WT and KO spheres averages 1 out of 5000 cells seeded. (n=7 trials each). B) WT and KO spheres average 100um diameter. (n=7 trials each)



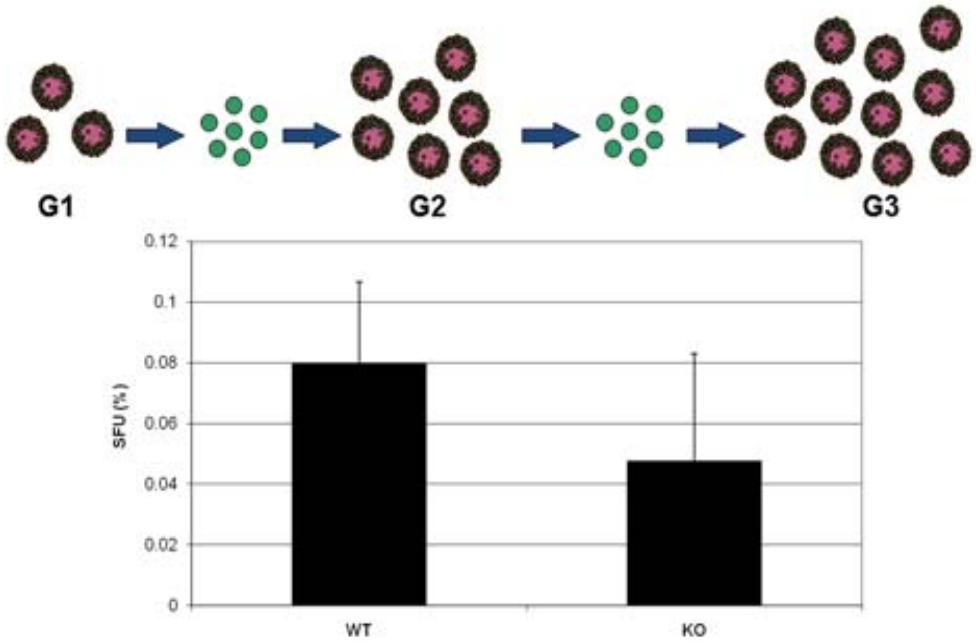


Fig 3: Effect of Nkx3.1 loss on self-renewal. Secondary spheres are formed (G2) by dissociating primary spheres and re-plating in matrigel. Nkx3.1 knockout spheres show a trend towards decrease in self renewal. N=2 trials. Additional trials might help to clarify if a statistically significant difference is seen.

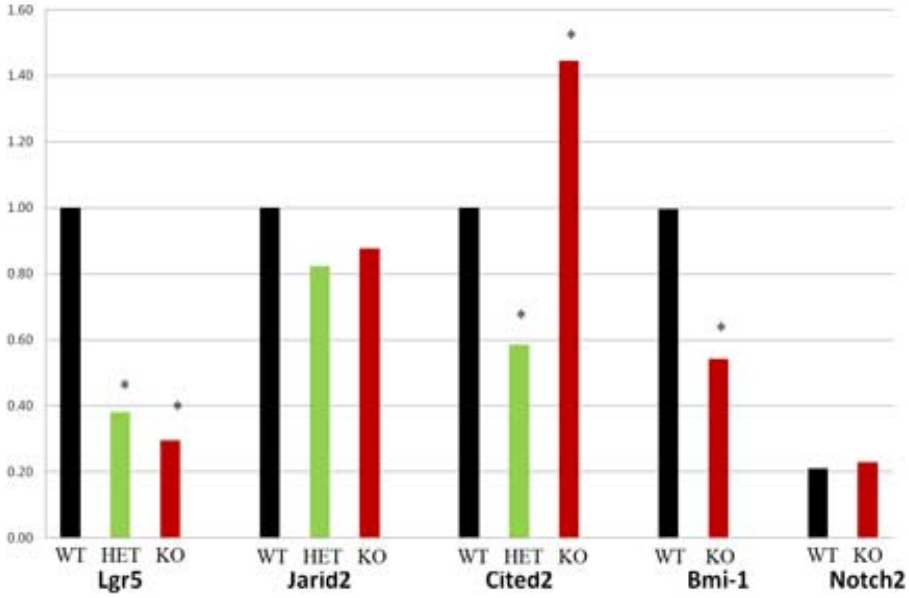


Fig. 4: Quantitative RT-PCR analysis for expression of stem cells markers in Nkx3.1 mutant prostate cells. Not changes in Lgr5 and Bmi1. * $P<0.05$

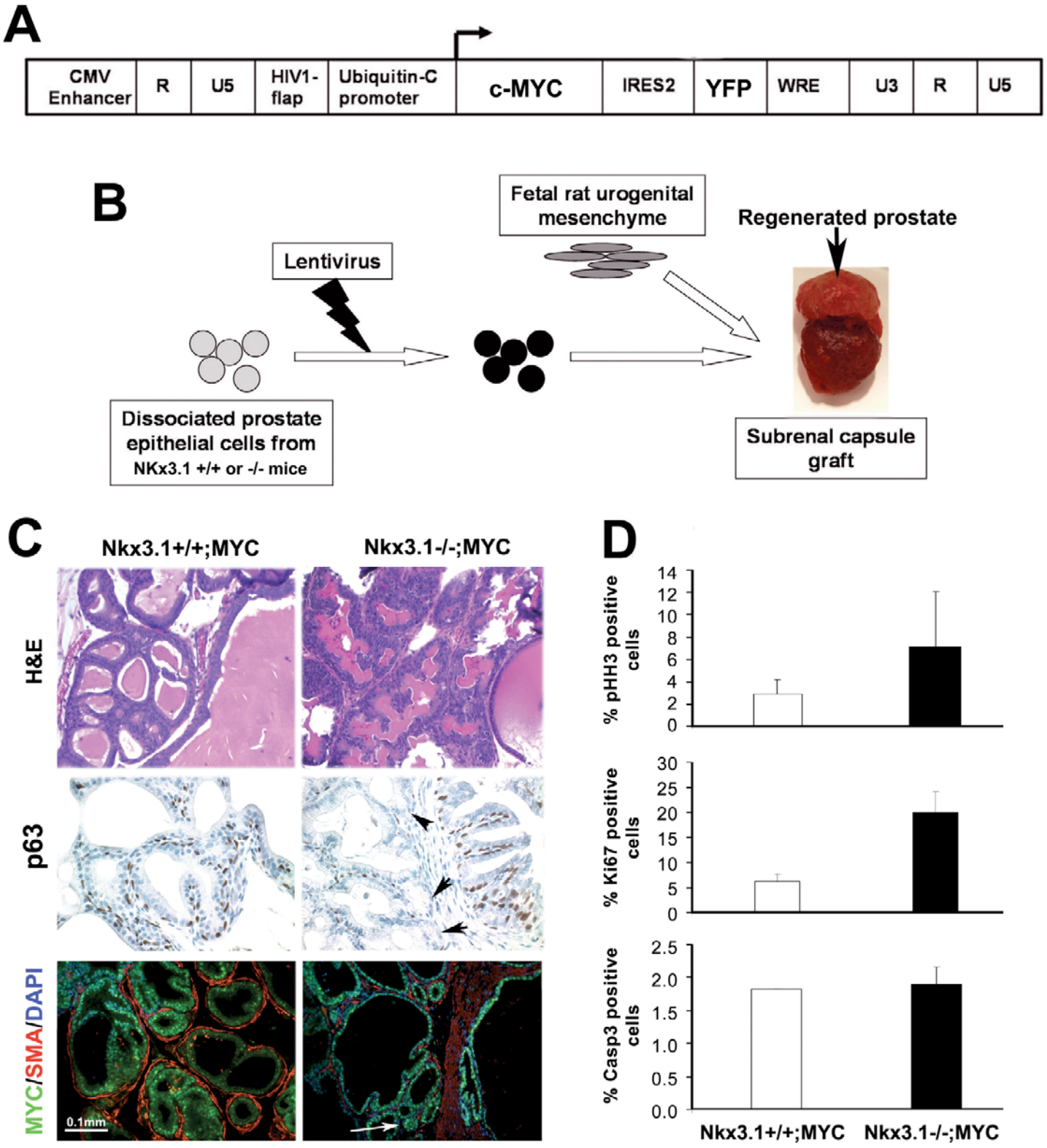


Fig. 5: Cooperativity between MYC and Nkx3.1-loss in vivo by prostate regeneration. A, Schematic of lentivirus expressing MYC and YFP. B, Outline of tissue recombination and subrenal capsule grafting. C, Histology, focal loss of basal cells (p63) and microinvasion (focal loss of smooth muscle actin, SMA) indicate progression in Nkx3.1-/-;MYC prostate grafts compared to Nkx3.1+/+;MYC grafts (N=3 each). D, Increased proliferation (phospho-histone H3, pHH3 and Ki67 staining) in Nkx3.1-/-;MYC grafts compared to Nkx3.1+/+;MYC grafts. Double staining with MYC was used to enable quantitation of proliferation in MYC-expressing glands. Apoptosis (activated- caspase3 staining) was unchanged. ***Note: Statistical analysis pending additional graft data.